# Genetic and biochemical characterization of an exopolygalacturonase and a pectate lyase from *Yersinia enterocolitica*[1]

**Ching-Hsing Liao, Larry Revear, Arland Hotchkiss, and Brett Savary**

**Abstract**: *Yersinia enterocolitica*, an invasive foodborne human pathogen, degrades polypectate by producing two depolymerizing enzymes, pectate lyase (PL) and polygalacturonase (PG). The gene encoding the PG activity, designated *pehY*, was located in a 3-kb genomic fragment of *Y. enterocolitica* ATCC 49397. The complete nucleotide sequence of this 3-kb fragment was determined and an open reading frame consisting of 1803 bp was predicted to encode a PG protein with an estimated $M_r$ of 66 kDa and pI of 6.3. The amino acid sequence of prePG showed 59 and 43% identity to that of the exopolygalacturonase (exoPG) of *Erwinia chrysanthemi* and *Ralstonia solanacearum*, respectively. The *Y. enterocolitica* PG overproduced in *Escherichia coli* was purified to near homogeneity using perfusion cation exchange chromatography. Analysis of the PG depolymerization products by high performance anion-exchange chromatography and pulsed amperometric detection (HPAEC-PAD) revealed the exolytic nature of this enzyme. The *Y. enterocolitica* PL overproduced in *E. coli* was also partially purified and the $M_r$ and pI were estimated to be 55 kDa and 5.2, respectively. HPAEC-PAD analysis of the PL depolymerization products indicated the endolytic nature of this enzyme. Southern hybridization analyses revealed that *pehY* and *pel* genes of *Y. enterocolitica* are possibly encoded in the chromosome rather than in the plasmid. Purified exopolygalacturonase (over 10 activity units) was unable to macerate plant tissues.

*Key words*: pectinase activities, human pathogen, HPLC analysis, *pehY* gene.

**Résumé** : *Yersinia enterocolitica*, un pathogène invasif de l'homme transmis par les aliments, dégrade le polypectate en produisant deux enzymes de dépolymérisation, soit la pectate lyase (PL) et la polygalacturonase (PG). Le gène responsable de l'activité PG, désigné *pehY*, a été localisé sur un fragment génomique de 3-kb chez *Y. enterocolitica* ATCC 49397. La séquence nucléotidique complète de ce fragment de 3-kb a été déterminée et il est prévisible qu'un cadre de lecture ouvert comprenant 1803 pb code une protéine PG dont le Mr est estimé à 66 kDa et le pI à 6.3. La séquence des acides aminés de préPG a révélé 59% d'identité avec l'exopolygalacturonase (exoPG) d'*Erwinia chrysanthemi* et 43% avec celle de *Ralstonia solanacearum*. La PG de *Y. enterocolitica*, surproduite dans *E. coli*, a été purifiée jusqu'à homogénéité apparente par chromatographie échangeuse de cations en perfusion. L'analyse des produits de dépolymérisation PG par chromatographie échangeuse d'anions à haute performance et la détection ampérométrique pulsée (HPAEC-PAD) a confirmé la nature exolytique de cette enzyme. La PL de *Y. enterocolitica*, surproduite dans *E. coli*, a aussi été partiellement purifiée et le Mr et le PI étaient respectivement de 55 Kda et 5.2. L'analyse HPAEC-PAD des produits de dépolymérisation PL a confirmé la nature endolytique de cette enzyme. L'hybridation Southern a montré que les gènes *pel* et *pehY* de *Y. enterocolitica* étaient possiblement localisés sur le chromosome plutôt que sur le plasmide. L'exoPG purifiée (plus de 10 unités d'activité) était incapable de causer la macération des tissus végétaux.

*Mots clés* : activité pectinase, pathogène humain, analyse HPLC, gène *pehY*.

[Traduit par la Rédaction]

## Introduction

*Yersinia enterocolitica* is an enteropathogen capable of producing gastroenteritis in humans similar to that caused by *Salmonella* and *Shigella* (Robins-Browne 1997). This pathogen occupies a broad range of environments and has been found in a wide variety of foods including pork, beef, poultry, and dairy products (Robins-Browne 1997). Although animals, particularly swine, are the predominant sources for *Y. enterocolitica*, this pathogen has been detected in raw vegetables (Beuchat 1996). Catteau et al. (1985) analyzed 58 samples of grated carrot and found that 27% of the samples were contaminated with *Yersinia*. Darbas et al. (1985) examined raw vegetables that were destined for school meals and

Received December 1, 1998. Revision received March 3, 1999. Accepted March 3, 1999.

**C.-H. Liao,[2] L. Revear, A. Hotchkiss, and B. Savary.** Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 600 East Mermaid Lane, Wyndmoor, PA 19038, U.S.A.

[1]Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over the others of a similar nature not mentioned.

[2]Author to whom all correspondence should be addressed (e-mail: cliao@arserrc.gov).

found that 50% of the samples were contaminated with *Yersinia* species. Although *Y. enterocolitica* is not generally considered a major pathogen of food safety concern, at least two yersiniosis outbreaks have been associated with consumption of contaminated plant products, including tofu (bean curd) (Tacket et al. 1985) and bean sprouts (Cover and Aber 1989). Fresh and minimally processed fruits and vegetables thus represent a potential site for the growth and transmission of *Y. enterocolitica* (Karapinar and Gonul 1992). Recently, several studies (reviewed in Beuchat 1996; Nguyen-the and Carlin 1994) have shown that foodborne human pathogens are able to survive and grow in minimally processed fruits and vegetables. However, it has not yet been determined if and how *Y. enterocolitica* may survive and grow on plants and minimally processed produce.

Among foodborne pathogens, *Yersinia* species are unique in their ability to degrade pectic components that are found naturally in plant cell walls. In 1975, von Riesen first reported that members of *Y. enterocolitica* and *Yersinia pseudotuberculosis*, which are pathogenic to animals, are capable of digesting calcium-stabilized polypectate gel. A follow-up study by Starr et al. (1977) further demonstrated that the pectolytic activity of *Yersinia* species stems mainly from the production of pectate lyase (PL). PL is commonly known to be produced by plant pathogens and is the principal factor responsible for the spoilage of fresh produce caused by various strains of pectolytic bacteria (Liao 1989). In addition to PL, Starr et al. (1977) also briefly noted the detection of polygalacturonase (PG) activities in culture fluids of a few *Yersinia* strains. However, this PG has never been isolated and characterized. Although the PLs produced by *Yersinia* strains have been investigated (Bagley and Starr 1979; Chatterjee et al. 1979), nothing is presently known about the PG produced by *Yersinia* species. Since *Yersinia* species are generally not considered natural inhabitants of plants, the reason for producing pectic enzymes by these human and animal pathogens remains a mystery. As the first step to understanding the ecological functions of pectinase from *Yersinia* species, we re-examined the pectinases, especially PG, produced by different strains of *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Yersinia kristensenii*. Also as a means of obtaining a large quantity of PG for biochemical characterizations, we cloned and sequenced a gene encoding the PG activity from *Y. enterocolitica*. We report here the first detailed account of the PG and the depolymerization pattern of the PL produced by a human-pathogenic strain of *Y. enterocolitica*.

## Materials and methods

### Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids used are listed in Table 1. For routine cultivation, *Yersinia* strains were grown in brain heart infusion (BHI) broth or agar (Difco Laboratories, Detroit, Mich.) at 28°C and *Escherichia coli* strains were grown in Luria broth (LB) or agar (Life Technologies, Gaithersburg, Md.) at 37°C. If needed, an enriched minimal salt (EMS) medium containing yeast extract (0.1%), Casamino acids (0.1%), glycerol (0.2%), and polygalacturonic acid (0.2%) at pH 7.1 was used. The minimal salt solution contained $K_2HPO_4$ (0.7%), $KH_2PO_4$ (0.2%), $MgSO_4{\cdot}7\ H_2O$ (0.02%), $(NH_4)_2SO_4$ (0.1%), and $CaCl_2$ (0.5 mM). A semisolid pectate (SSP) medium (Liao et al. 1996) was used to detect the pectolytic activity of *Yersinia* species, and *E. coli* strains carrying the pectinase gene. If required, antibiotics were added at the following concentrations: 50 μg ampicillin·$mL^{-1}$, 25 μg tetracycline·$mL^{-1}$, and 50 μg kanamycin·$mL^{-1}$.

### Enzyme preparation and purification

Bacterial cells grown in various culture media were fractionated, and enzyme activities in the extracellular, periplasmic, and cytoplasmic fractions were determined as previously described (Liao et al. 1996). Briefly, bacterial cells were separated from the culture medium by centrifugation (10 000 × *g* for 10 min) and the supernatant obtained was used to assay extracellular activity. Cell pellets were washed, resuspended in 0.2 M Tris–HCl (pH 8.0), and then used to prepare the periplasmic fluids using procedures previously described (Witholt et al. 1976). The total activity in the periplasmic and cytoplasmic fractions was considered the cell-bound activity. For purification of enzymes, *E. coli* cells carrying the *pel* gene coding for PL activity or the *pehY* gene coding for PG activity of *Y. enterocolitica* (in the construct pYEII-5-H192 or pYEII-4-H192, respectively) were grown in EMS medium at 37°C for 40 h. Osmotic shock fluids were prepared as described above and concentrated by ultrafiltration using the Amicon PM 10 membrane (Schleicher & Schuell, Keene, N.H.). The concentrated osmotic fluid (2.5 mg protein·$mL^{-1}$) containing PG was applied into a Poros 20 HS cation exchange column (4.6 × 10 mm; Perseptive Biosystems) in 20 mM MES buffer (pH 6.0) at a flow rate of 5 mL/min. Following a pre-elution step with 100 mM NaCl, PG was eluted isocratically with 225 mM NaCl. Similarly, concentrated periplasmic fluids containing PL were injected onto a Poros PI anion exchange column with 10 mM Bis–Tris buffer (pH 6.0). PL activity was eluted in the void volume, and the active fractions were pooled and concentrated by ultrafiltration.

### Enzyme assays

PL activity was assayed in a reaction mixture (0.4-mL volume) containing 100 mM Tris–HCl (pH 8.0), 1 mM $CaCl_2$, 0.2% w/v polygalacturonic acid, and enzyme sample as previously described (Liao 1989). One unit (U) of activity was defined as the amount of enzyme that caused an increase of 1.0 absorbance unit/min at 232 nm and 20°C. PG activity was assayed by measuring the increase in reducing sugars in a reaction mixture (pH 5.3) containing 0.2% polygalacturonic acid, 60 mM sodium acetate, 7.5 mM EDTA, and 0.12 M NaCl by the copper-arsenomolybdate method (Collmer et al. 1988). One unit (U) of activity was defined as the amount of enzyme that caused an increase of 1.0 absorbance unit/h at 500 nm under the conditions previously described (Collmer et al. 1988).

### Tissue-maceration assays

The ability of bacterial cells or purified enzyme samples to macerate plant tissue was tested on potato tuber slices, bell pepper, and cucumber. Methods for preparing plant materials and bacterial cell suspensions for inoculation have been described previously (Liao and Wells 1987). Surface-sterilized potato tuber discs, the inner parts of bell pepper, or cucumber slices were placed on 0.6% water agar plates. Each slice was inoculated with 5–10 μL of a bacterial suspension ($10^{10}$ colony-forming units (CFU)·$mL^{-1}$) or 0.1–10 U of enzyme samples and then incubated at 28°C for 2–24 h for purified enzymes and 3 days for live bacteria.

### Polyacrylamide gel electrophoresis and isoelectric focusing

Sodium dodecyl sulfate (SDS) – polyacrylamide gel electrophoresis (SDS–PAGE) was conducted in BioRad 12% Ready Gels (BioRad Laboratories, Hercules, Calif.) using Laemmli buffer solution (Laemmli 1970). Isoelectric focusing (IEF) was done in

**Table 1.** Bacterial strains and plasmids.

| Bacterial strains | Relevant properties | Reference |
|---|---|---|
| *Yersinia enterocolitica* | | |
| ATCC 35669 | Wild type | ATCC |
| ATCC 49397 | Wild type; clinical isolate | ATCC |
| JB580V | Wild type; clinical isolate | Badger and Miller 1995 |
| *Y. pseudotuberculosis* | | |
| ATCC 29833 | Wild type; isolated from turkey | ATCC |
| *Y. kristensenii* | | |
| ATCC 33639 | Wild type; isolated from hare | ATCC |
| Plasmids | | |
| pYEII-1 to pYEII-6 | Primary cosmid clones carrying *Y. enterocolitica pel* gene (pYEII-1, pYEII-3, and pYEII-5) and clones carrying *pehY* gene (pYEII-2, pYEII-4, and pYEII-6) | This study |
| pYEII-4-H192 | 7.6-kb *Hin*dIII fragment from pYEII-4 cloned into pUC19, contains an *pehY* gene | This study |
| pYEII-4-E30B and pYEII-4-E30BA | 3-kb *Eco*RI fragment from pYEII-4-H192 cloned into pUC19 in opposite orientations | This study |
| pYEII-41 (to pYEII-46) | *Tn*5 insertions into the *pehY* locus in pYEII-4, nonpectolytic, $Tc^r$ $Km^r$ | This study |
| pYEII-5-H192 | 5-kb HindIII fragment from pYEII-5 cloned into pUC19, contains a *pel* gene | This study |
| pYEII-5-H1921 (to pYEII-5-H1926) | *Tn*5 insertions into the *pel* locus of pYEII-5-H192, nonpectolytic | This study |
| pPELY14 | pUC19 contains *Y. pseudotuberculosis pelY* | Manulis et al. 1988 |
| Phage λ467 | Contains *Tn*5; used for mutagenesis | Ruvun and Ausubel 1981 |

**Notes**: PL, pectate lyase; PG, polygalacturonase; $Tc^r$, resistance to tetracycline; $Km^r$, resistance to kanamycin; *pel*, gene coding for PL; *pehY*, gene coding for PG activity.

premade polyacrylamide gels (PAG plates, pH 3.5–9.5, Pharmacia-LKB Biotechnology, Piscataway, N.J.) as previously described (Liao 1989). After electrophoresis, gels were stained with Coomassie Blue for detection of proteins or subjected to overlay activity staining techniques for detection of enzyme activities under the conditions previously described (Liao 1989). Prior to the detection of enzyme activities for protein bands in SDS – polyacrylamide gel, the gel was rinsed in 50 mM Tris–HCl (pH 7.0) buffer to remove SDS. Protein concentrations in the samples were determined by measuring the absorbance at 280 nm or by the Bradford procedure included in the BioRad Protein Assay Kit (BioRad Laboratories). Proteins were electro-blotted from SDS–PAGE gel to polyvinylidene difluoride membranes for N-terminal amino acid sequencing using a BioRad Mini Trans-blot transfer cell. Transfer was accomplished in 10 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS) buffer (pH 10) containing 10% v/v methanol running at 200 mA for 50 min.

## Analysis of enzyme depolymerization patterns by high performance anion-exchange chromatography and pulsed amperometric detection

The PG substrate solution containing 0.2% PGA, 0.12 M NaCl, 0.05 M $CH_3COONa$ (pH 5.3), and the PL substrate solution containing 0.2% PGA, 0.1 M N-tris-methyl-3-aminopropanesulfonic acid (TAPS) buffer (Sigma), and 1 mM $CaCl_2$ (pH 9.5) were used. Both substrate solutions were filter (0.2 μm) sterilized and the final enzyme concentrations used were 0.2 $U \cdot mL^{-1}$. Periodically, aliquots (1 mL) were removed from assay solutions and reaction products analyzed by high performance anion-exchange chromatography and pulsed amperometric detection (HPAEC-PAD) as reported previously (Hotchkiss and Hicks 1990). Oligogalacturonic acid separation was conducted using a nonlinear potassium oxalate buffer (pH 6) mobile phase, a CarboPAC PA1 column (Dionex) and post-column addition of 500 mM KOH (Hotchkiss and Hicks 1990). Chromatograms were collected and analyzed with a Chrom Perfect Direct (Justice Innovations) chromatography data system that included a DT2804 A-D board.

## Recombinant DNA technologies

Genomic DNA of *Y. enterocolitica* ATCC 49397 was partially digested with *Sau*3A and genomic fragments of 15- to 30-kb in size were ligated to *Bam*HI-digested pLAFR3 (Staskawicz et al. 1987). The ligation sample was packaged in vitro and *E. coli* transductants obtained were screened for pectolytic activity on semi-solid pectate (SSP) medium (Liao et al. 1996). λ-mediated *Tn*5 mutagenesis was conducted in accordance with the methods previously described (Ruvun and Ausubel 1981). *E. coli* cells carrying a plasmid containing the *pel* (in pYEII–5-H192) or the *pehY* gene (in pYEII-4) were infected with λ 467 at a multiplicity of infection of 1.0. *Tn*5-generated mutants of pYEII-5-H192 and pYEII-4 in *E. coli* were screened for pectolytic activity on SSP medium, and nonpectolytic derivatives were selected for further analysis. Southern blot analysis was performed according to the standard procedures (Sambrook et al. 1989). DNAs were labeled with dideoxygenin and detected by using the protocols described in the Genius DNA Labeling and Detection Kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Genomic DNA of *Yersinia* species were extracted by the method previously described (Nakajima et al. 1992). Plasmid and chromosomal DNAs were separated by agarose gel electrophoresis and then blotted onto a nitrocellulose membrane. The blots were probed with a specific *pel* or *pehY* fragment to determine if the *pel* or *pehY* gene was located in the chromosome or plasmid.

## DNA and N-terminal protein sequencing

The 3-kb genomic fragment containing the *Y. enterocolitica pehY* gene was sequenced on both strains by the standard dideoxy chain termination method. Primers were synthesized, and sequencing reactions were conducted at Labstand Lab., Ltd. (Gaithersburg, MD) by using ThermoSequenase with $^{32}P$-terminators. Sequence analyses were performed using the PC/GENE software programs (Release 6.8) from Intelligenetics, Inc. (Mountain View, Calif.). N-terminal amino acid sequence of the PG protein on the electro-blotted membrane was determined at the Macromolecular Core Facility, Pennsylvania State University (Hershey Medical Center, Hershey, Penn.). Complete sequence of the 3-kb fragment contain-

**Table 2.** Production of PL and PG by different species of *Yersinia*.

| *Yersinia* strains | PL Total activity ($U/10^{10}$ cells) | PL % cell-bound activity | PG Total activity ($U/10^{10}$ cells) | PG % cell-bound activity |
|---|---|---|---|---|
| *Y. enterocolitica* | | | | |
| ATCC 35669 | 2.8±0.6 | 85 | 0.4±0.1 | 88 |
| ATCC 49397 | 10.3±0.5 | 79 | 2.1±0.4 | 86 |
| JB580v | 9.4±0.7 | 86 | 1.9±0.3 | 78 |
| *Y. pseudotuberculosis* | 0.9±0.2 | 75 | ND | — |
| *Y. kristensenii* | 0.7±0.3 | 73 | ND | — |

**Notes**: One unit (U) of PL is defined as the amount of enzyme that causes an increase of 1 absorption unit/min at 232 nm and 20°C under the assay conditions described in Materials and methods. One U of PG is defined as the amount of enzyme that causes an increase of 1 absorption unit/h at 500 nm and 28°C under the conditions described in Materials and methods. % Cell-bound activity = (activity in periplsamic and cytoplsamic fractions / total activity in culture fluid, periplasm, and cytoplasm) × 100%. The values are means ± SD for three experiments with two duplicates in each experiment. ND, not detected.

ing the *Y. enterocolitica pehY* gene has been submitted to the GenBank (National Center for Biotechnology Information, Bethesda, Md.) with the accession number AF059505.

## Results and discussion

### Pectic enzyme production by *Yersinia*

All three *Y. enterocolitica* strains and one strain each of *Y. pseudotuberculosis* and *Y. kristensenii* included in this study displayed various degrees of pectolytic activity on SSP medium. Production of PL or PG was detected in bacterial cells grown in rich broth media including BHI and LB. However, three- and five-fold higher levels of PL or PG activity, respectively, were observed with cells grown in EMS medium. Previously, Chatterjee et al. (1979) reported that a clinical strain of *Y. enterocolitica* synthesized higher levels of PL activity when grown in the presence of gluconate, glycerol, or polygalacturonate, and considerably less activity when grown in glucose. Here, we found that higher levels of PG activity were in minimal salt medium than in rich media. Results (Table 2) show that production of PL is common among all five *Yersinia* strains. However, production of PG was detected in cultures of three *Y. enterocolitica* strains but not in cultures of *Y. pseudotuberculosis* and *Y. kristensenii* strains. The amounts of PL and PG produced by *Yersinia* spp. varied with the strains; the highest level of activity was detected in cultures of two *Y. enterocolitica* strains (ATCC 49397 and JB580v). Furthermore, activities of PL and PG were detected mainly (over 70% of total activity) in the cell-bound fraction (Table 2). Bagley and Starr (1979) found the *Y. enterocolitica* PL largely in the cell-bound fraction. Here we found that the *Y. enterocolitica* PG was also located intracellularly.

### Cloning and analysis of *pehY* gene

A genomic library consisting of 1500 *E. coli* $Tc^r$ clones were screened for pectolytic activity on SSP medium. Six pectolytic clones (pYEII-1 to pYEII-6) were isolated and characterized. Enzyme activity measurements of *E. coli* cells carrying these primary clones showed that pYEII-1, pYEII-3, and pYEII-5 encoded for PL, whereas pYEII-2, pYEII-4, and pYEII-6 encoded for PG. When these six pectolytic clones were probed with a specific *pel* fragment (the internal 0.7-kb *Eco*RV–*Hin*dIII region from pPelY14) from *Y. pseudotuberculosis* (Manulis et al. 1988), *Y. enterocolitica pel* homologs were detected in pYEII-1, pYEII-3, and pYEII-5, but not in pYEII-2, pYEII-4, and pYEII-6. This result confirmed that the *pel* was present in pYEII-1, pYEII-3, or pYEII-5, and the *pehY* gene coding for PG activity was possibly present in pYEII-2, pYEII-4, and pYEII-6. To determine if only one copy of *pehY* gene was present in the clone, pYEII-4 was mutagenized with λ 467 (containing *Tn5*) and six nonpectolytic mutants (pYEII-41 to pYEII-46) randomly selected were found to contain *Tn5* in a 3-kb *Eco*RI fragment. To confirm that this 3-kb *Eco*RI fragment contains the *pehY* gene, the 3-kb *Eco*RI fragment was cloned into pUC19 in two opposite orientations to form pYEII-4-E30A and pYEII-4-E30B. *Escherichia coli* cells carrying either pYEII-4-E30A or pYEII-4-E30B were able to synthesize about the same level of PG, suggesting that the PG synthesis is possibly initiated from the native *pehY* gene promotor.

The *pehY*-containing 3-kb fragment was sequenced and an open reading frame (ORF) consisting of 1803 bp was identified (GenBank accession No. AF059505). Preceding the putative translational initiation codon, a potential ribosome-binding site (AAGG) and a putative promotor region (bases 735–762) were identified. The hairpin structure frequently associated with the transcriptional termination in bacterial pectinase genes (Liao et al. 1996) was not found, but two overlapping inverted repeats were found 27 bp downstream of the stop codon. The ORF was predicted to encode a protein consisting of 601 amino acids (aa) with an estimated $M_r$ of 66 kDa and pI of 6.3. Protein sequence analysis revealed that this protein shares 59% identity in aa sequences to the exopolygalacturonase (exoPG) of *Erwinia chrysanthemi* (He and Collmer 1990) (Fig. 1) and 43% identity to the exoPG of *Ralstonia solanacearum* (Huang and Allen 1997). However, the predicted *Y. enterocolitica* PG showed very low identity in aa sequence (less than 15%) to endoPGs of *R. solanacearum*, *Erwinia carotovora,* and *Agrobacterium vitis* (Herlache et al. 1997). A putative signal peptide (29 aa residues) and signal peptidase cleavage site were identified (Fig. 1). With the exception of the first alanine residue, 14 of the remaining 15 aa in the N-terminus were confirmed by N-terminal sequencing (Fig. 1). This is the first report of the

**Fig. 1.** Amino acid sequence alignment of predicted exopolygalacturonase proteins from *Y. enterocolitica* ATCC 49397 (PEHY_YEREN) and *Erwinia chrysanthemi* EC16 (PEHX_ERWCH). The underlining indicates the putative signal peptide sequences and the arrows indicate the putative signal peptidase cleavage sites. Dots show the N-terminal amino acid residues of the mature protein confirmed by protein sequencing.

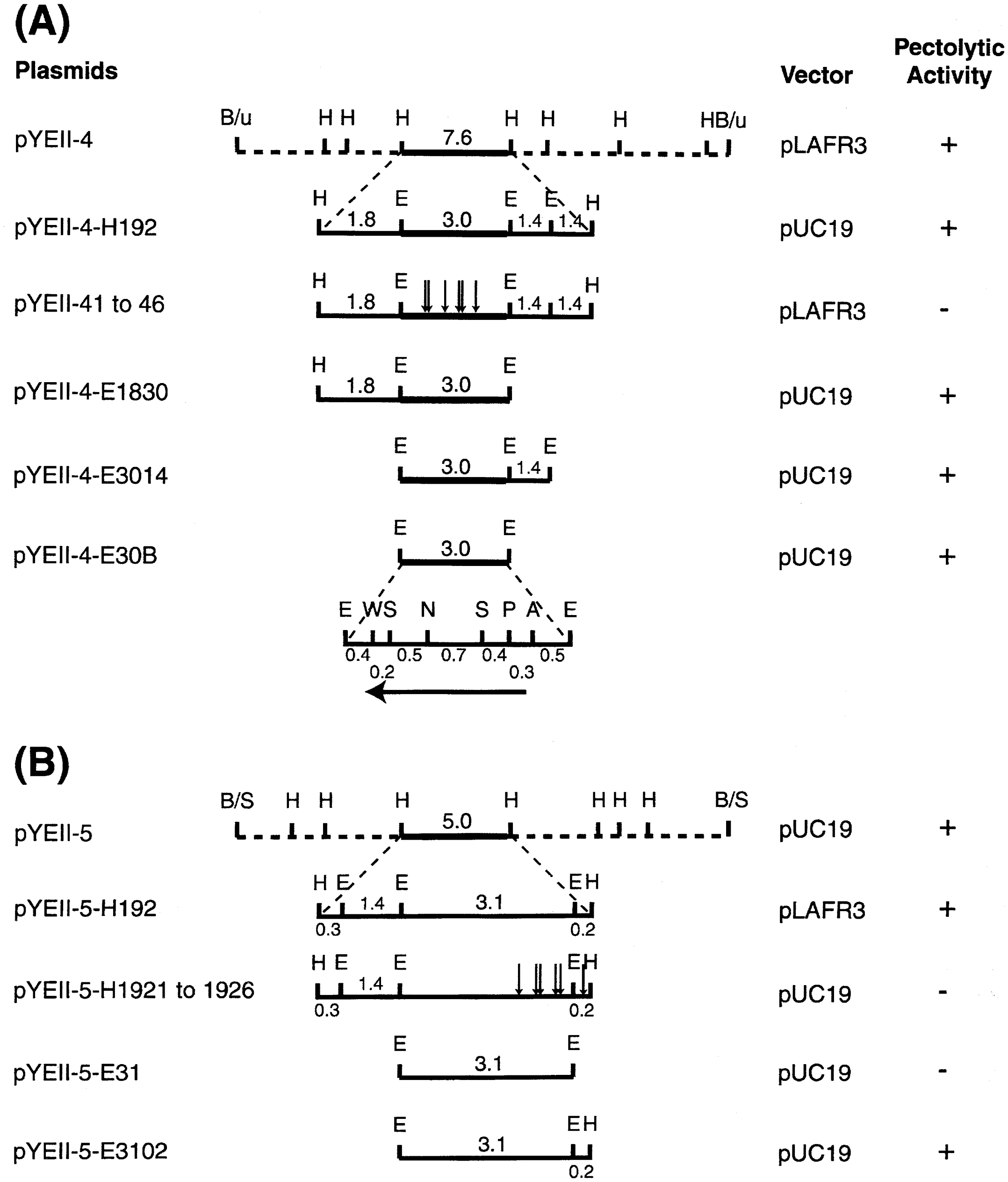
```
PEHY_YEREN- MQAQLQRPRTTGMLVIMASLMVGTPMAMAAKSSSLDAPQQLQVPTLAYDE -50
PEHX_ERWCH- MKVITFSRRSALASIVATCLM-----STPALAATAQAPQKLQIPTLSYDD -45

PEHY_YEREN- SSIVLVWKAPEDTRKIVDYQIFSAGKLLGKASDNNDNFSPAKPYIDHFYA -100
PEHX_ERWCH- HSVMLVWDTPEDTSNITDYQIYQNGQLIGLASQNNDKNSPAKPYISAFYK -95

PEHY_YEREN- NDKDNFQHKIVMQNFTVIGLKPETSYQFTVKAQYADGSLSVASKPITAKT -150
PEHX_ERWCH- SDAANFHHRIVLQNAKVDGLKAGTDYQFTVRTVYADGTTSNDSNTVTTTT -145

PEHY_YEREN- SAKPQIVNVRDFGAIDDGKTLNTKAIQQAIDSCKPGCRVEIPAGTYKSGA -200
PEHX_ERWCH- TAVPKVINITQYGAKGDGTTLNTSAIQKAIDACPTGCRIDVPAGVFKTGA -195

PEHY_YEREN- LWLKSDMTLNLQAGAILLGSENPDDYPAGYRLYPYSTIERPASLINAIDP -250
PEHX_ERWCH- LWLKSDMTLNLLQGATLLGSDNAADYPDAYKIYSYVSQVRPASLLNAIDK -245

PEHY_YEREN- NNSKPGTFRNIRITGSGVIDGNGWLRAKTAEITDELGRSLPQYVASKNSK -300
PEHX_ERWCH- NSSAVGTFKNIRIVGKGIIDGNGWKR--SADAKDELGNTLPQYVKSDNSK -293

PEHY_YEREN- VHEDGILAKNQVEKAVSDGMDLKNAYGQRRSSLMTLRGVENVYLAGFTVR -350
PEHX_ERWCH- VSKDGILAKNQVAAAVATGMDTKTAYSQRRSSLVTLRGVQNAYIADVTIR -343

PEHY_YEREN- NPAFHGIMNLENHNVVANGLIHQTYDANNGDGIEFGNSQNVMVFNNFFDT -400
PEHX_ERWCH- NPANHGIMFLESENVVENSVIHQTFNANNGDGVEFGNSQNIMVFNSVFDT -393

PEHY_YEREN- GDDCINFAAGTGEKAQEQEPMKGAWLFNNYFRMGHGAIVTGSHTGAWIED -450
PEHX_ERWCH- GDDSINFAAGMGQDAQKQEPSQNAWLFNNFFRHGHGAVVLGSHTGAGIVD -443

PEHY_YEREN- ILAENNVMYLTDIGLRAKSTSTIGGGARNVTFRNNAMRDLAKQVMVMTLD -500
PEHX_ERWCH- VLAENNVITQNDVGLRAKSAPAIGGGAHGIVFRNSAMKNLAKQAVIVTLS -493

PEHY_YEREN- YADSNANIDYPPAKIPAQFYDFTLKNVTVDNSTGKNPSIEIKGDTANKAW -550
PEHX_ERWCH- YADNNGTIDYTPAKVPARFYDFTVKNVTVQDSTGSNPAIEITGDSSKDIW -543

PEHY_YEREN- HRLVHVNNVQLNNVTPTAIMDLRDSEFNKVTFTELRGDT-PWHFSEVKKC -599
PEHX_ERWCH- HSQFIFSNMKLSGVSPTSISDLSDSQFNNLTFSNLRSGSSPWKFGTVKNV -593

PEHY_YEREN- QG        -601

PEHX_ERWCH- TVDGKTVTP -602
```

cloning of a *pehY* gene from *Yersinia* species and sequence analysis indicates that this gene encodes an exoPG.

## Analysis of PG protein

The PG was purified from concentrated osmotic shock fluids of *E. coli* cells carrying pYEII-4-H192 (Fig. 2) by cation exchange chromatography. Analysis of purified enzyme samples by SDS–PAGE showed that the *Y. enterocolitica* PG overproduced in *E. coli* had been purified to near homogeneity (Fig. 3A). A single PG band with an estimated $M_r$ of 66 kDa (Fig. 3A, lane 3) was confirmed by overlay enzyme activity staining. The $M_r$ of PG estimated by SDS–PAGE was approximately 3 kDa larger than that predicted from the protein sequence. Previously, He and Collmer (1990) also reported that the $M_r$ of an exoPG from *Erwinia chrysanthemi* was slightly smaller than that actually observed in the SDS – polyacrylamide gel. Purified PG was also analyzed by IEF electrophoresis and enzyme activity staining. The pI of PG was estimated to be 6.6, which was about 0.3 unit lower than that predicted from the sequence analysis. Purified PG was used to digest polygalacturonic acid, and the resulting enzymatic products were analyzed by HPAEC-PAD. Figure 4A shows that the predominant product detected after 23–144 h of digestion is dp 2 oligogalacturonic acid (dp = degree of depolymerization). This result combined with the sequence analysis indicates that *Y. enterocolitica* PG is indeed an exoPG. The *A. vitis* PG (only known bacterial PG with an acidic pI) also produce predominately dp 2 and 3 plus lower levels of larger oligogalacturonate (Herlache et al. 1997). He and Collmer (1990) suggested that the exoPG of *Erwinia chrysanthemi* may play a role in bacterial nutriention and in induction of other pectinase production. It remains to be determined if the *Y. enterocolitica* exoPG serves the same purpose and may be

**Fig. 2.** Restriction maps of cloned DNA fragments containing the *Y. enterocolitica* ATCC 49397 *pehY* gene encoding the exopolygalacturonase activity (A) and DNA fragments containing the *pel* gene encoding the pectate lyase activity (B). The numbers above the lines indicate the sizes of fragments in kilobases (kb) and the arrow indicates the coding region and possible direction of transcription. The vertical arrows denote the sites of *Tn5* insertions. B, *Bam*HI; H, *Hin*dIII; E, *Eco*RI; A, *Ava*I; S, *Stu*I; N, *Nde*I; P, *Sph*I; W, *Alw*NI; U, *Sau*3A; F, *Afl*III.




required for the survival and growth of this human pathogen in plants.

### Analysis of *pel* gene and PL product

The 5-kb *Hin*dIII fragment from pYEII-5 was cloned into pUC19 to form pYEII-5-H192 (Fig. 2B). pYEII5-H192 was able to direct the synthesis of high levels of PL in *E. coli*. Analysis of six nonpectolytic mutants of pYEII-5-H192 generated by *Tn5* mutagenesis revealed that *Tn5* insertions in these nonpectolytic mutants (pYEII-5-H1921-1926) were either in the 3.1- or 0.2-kb *Eco*RI fragment, indicating that the *Y. enterocolitica pel* is contained within these two *Eco*RI fragments (Fig. 2B). The *pel* genes of *Y. enterocolitica* and *Y. pseudotuberculosis* are related as indicated by Sourthern blot analyses. However, Manulis et al. (1988) showed that the *Y. pseudotuberculosis pel* gene shares very low sequence identities with the *pel* genes of soft-rotting *Erwinia* and *Pseudomonas* (Liao et al. 1996). The *Y. enterocolitica* PL partially purified from osmotic shock fluids of *E. coli* cells carrying pYEII-5-H192 was also analyzed by SDS–PAGE, IEF electrophoresis, and enzyme activity staining (Fig. 5). The $M_r$ and pI of *Y. enterocolitica* PL was estimated to be 55 kDa and 5.2, respectively, which were close to those reported for *Y. pseudotuberculosis* PL (Manulis et al. 1988).

**Fig. 3.** Analysis of the exoPG of *Y. enterocolitica* overproduced in *E. coli* by SDS–PAGE (A) and by isoelectric-focusing and activity staining (B). (A) Lane 1, molecular mass markers; lane 2, concentrated osmotic shock fluid from *E. coli* cells carrying pYEII-H-192 (containing the gene encoding the exoPG activity); lane 3, purified exoPG. (B) Activity-stained IEF gel showing the estimated pI of exoPG.

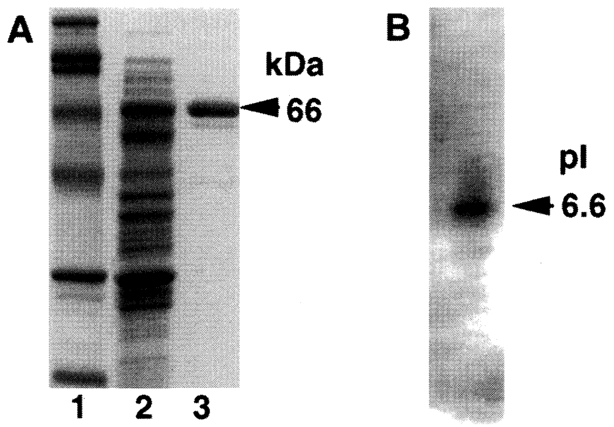
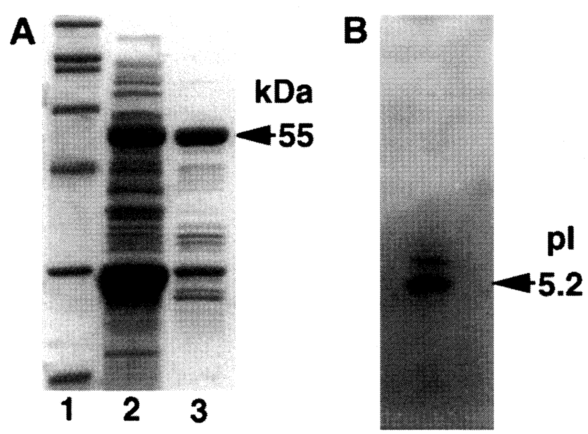
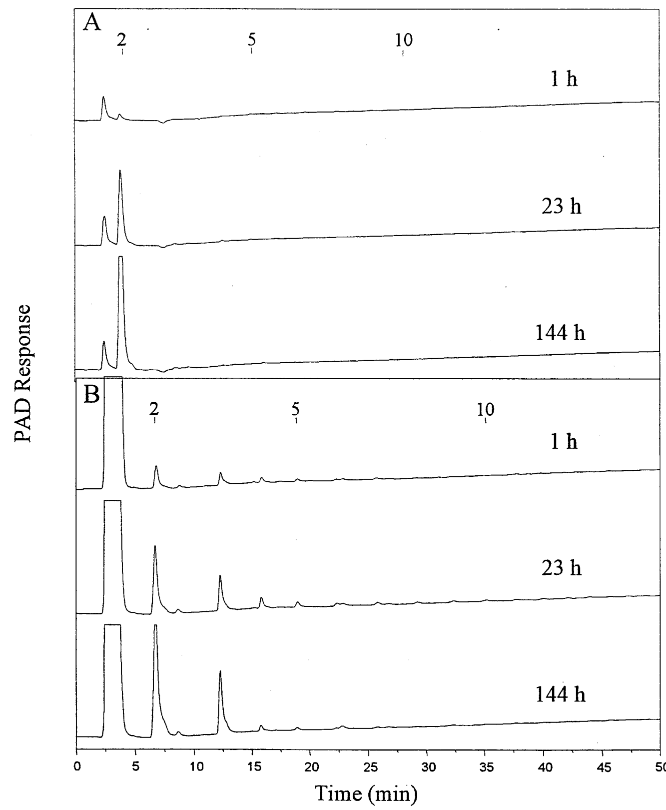



**Fig. 5.** Analysis of *Y. enterocolitica* PL overproduced in *E. coli* by SDS–PAGE (A) and by a combination of isoelectric focusing and activity staining (B). (A) Lane 1, molecular mass markers; lane 2, concentrated osmotic shock fluid from *E. coli* carrying pYEII-5-H192 (containing the gene encoding PL activity); lane 3, partially purified PL sample. The arrow indicates the PL band (as determined by activity staining) with an estimated $M_r$ of 55 kDa. (B) Activity-stained IEF gel showing the PL activity band with an estimated pI of 5.2.




Analysis of PL depolymerization patterns by HPAEC-PAD (Fig. 4B) shows that the *Y. enterocolitica* PL generated predominately unsaturated dp 2 and 3 oligogalacturonic acids. Small amounts of larger oligogalacturonic acids were produced during the PL depolymerization time course (Fig. 4B) demonstrating that this is an endolytic enzyme. The depolymerization pattern of *Yersinia* PL appears to be similar to that previously reported for *E. chrysanthemi* PLa (Preston et al. 1992).

**Fig. 4.** Analysis of the substrate depolymerization patterns of *Y. enterocolitica* pectinases overproduced in *E. coli* by high performance anion-exchange chromatography and pulsed amperometric detection systems (HPAEC-PAD).
(A) Exopolygalacturonase. (B) Endo-pectate lyase. The numbers above the peaks refer to the degree of polymerization of saturated (A) and unsaturated (B) oligogalacturonic acid released by each pectinase. The large void peak (2.4 min) in the endo-pectate lyase chromatograms is due to the TAPS buffer in the assay substrate solution. The HPAE-PAD chromatograms of 1, 23, and 144 h samples during the depolymerization time course are shown.




## Localization of *pel* and *pehY* genes in the genome of *Yersinia*

Endogenous plasmids (70–75 kb) were readily detected in two ATCC strains of *Y. enterocolitica* and *Y. pseudotuberculosis* included in the study. To determine if the pectinase gene was located in the chromosome or in the plasmid, total genomic DNAs were prepared from all four ATCC *Yersinia* strains. Following separation in agarose gel, plasmid and chromosomal DNA were probed with a specific *pel* fragment (the internal 0.7-kb *Eco*RV-*Hin*dIII fragment from pPelY14) or a specific *pehY* fragment (the internal 1.1-kb *Sph*I-*Nde*I fragment from pYEII-4-E30B). *pel* homologs were found in the chromosomal fraction in all four strains. However, *pehY* homologs were found in the chromosomal fraction in two *Y. enterocolitica* strains but not in *Y. kristensenii* and *Y. pseudotuberculosis*. The *pel* and *pehY* genes of all four ATCC *Yersinia* strains are possibly encoded in the chromosome rather than in the virulence plasmid.

### Maceration of plant tissue

None of the five *Yersinia* strains examined in the study caused maceration of potato tuber slices. Manulis et al. (1988) reported that purified PL from *Y. pseurotuberculosis* macerated cucumber tissue about 1000 times less efficiently than did PLe of *Erwinia chrysanthemi*. In this study, we were able to obtain a large quantity of purified exoPG from recombinant *E. coli* cells carrying the *pehY* gene to study the tissue-macerating activity of this enzyme. We found that 10 U of purified exoPG or partially purified PL was unable to cause detectable maceration in potato tuber, bell pepper, and cucumber slices after incubation at 28°C for 24 h. By comparison, 1.0 U of purified PL from *P. viridiflava* (Liao 1989) caused visible maceration of potato tuber slices, bell pepper, and cucumber within 5 h. This study demonstrates that the *Yersinia* exoPG overproduced in *E. coli* does not cause maceration of plant tissue. Inability of the *Yersinia* PL and exoPG to induce tissue-maceration is probably due to their intracellular location, low pI, or exolytic mode of action. It remains to be determined, however, if production of PL and PG by *Yersinia* species is required simply for catabolic functions (Chatterjee et al. 1979).

## References


Badger, J.L., and Miller, V.L. 1995. Role of RpoS in survival of *Yersinia enterocolitica* to a variety of environmental stresses. J. Bacteriol. **177**: 5370–5373.

Bagley, S.T., and Starr, M.P. 1979. Characterization of intracellular polygalacturonic acid *trans*-eliminase from *Klebsiella oxytoca, Yersinia enterocolitica,* and *Erwinia chrysanthemi*. Curr. Microbiol. **2**: 381–386.

Beuchat, L.R. 1996. Pathogenic microorganisms associated with fresh produce. J. Food Prot. **59**: 204–216.

Catteau, M., Krembel, C., and Wauters, G. 1985. *Yersinia enterocolitica* in raw vegetables. Sci. Aliments, **5**: 103–106.

Chatterjee, A.K., Buchanan, G.E., Behrens, M.K., and Starr, M.P. 1979. Synthesis and excretion of polygalacturonic acid trans-eliminase in *Erwinia*, *Yersinia*, and *Klebsiella* species. Can. J. Microbiol. **25**: 94–102.

Collmer, A., Ried, J.L., and Mount, M.S. 1988. Assay methods for pectic enzymes. Methods Enzymol. **161**: 329–335.

Cover, T.L., and Aber, R.C. 1989. *Yersinia enterocolitica.* N. Engl. J. Med. **321**: 16–24.

Darbas, H., Riviere, M., and Obertic, J. 1985. *Yersinia* in refrigerated vegetables. Sci. Aliments **5**: 81–84.

He, S.Y., and Collmer, A. 1990. Molecular cloning, nucleotide sequence, and marker exchange mutagenesis of the exo-poly-α-D-galacturonosidase-encoding *pehX* gene of *Erwinia chrysanthemi* EC16. J. Bacteriol. **172**: 4988–4995.

Herlache, T.C., Hotchkiss, A.T. Jr., Burr, T.J., and Collmer, A. 1997. Characterization of the *Agrobacterium vitis pehA* gene and comparison of the encoded polygalacturonase with the homologous enzymes from *Erwinia carotovora* and *Ralstonia solanacearum*. Appl. Environ. Microbiol. **63**: 338–346.

Hotchkiss, A.T., and Hicks, K.B. 1990. Analysis of oligogalacturonic acids with 50 or fewer residues by high-performance anion-exchange chromatography and pulsed amperometric detection. Anal. Biochem. **184**: 200–206.

Huang, Q., and Allen, C. 1997. An exo-poly-α-galacturonosidase, *pehB*, is required for wild-type virulence of *Ralstonia solanacearum*. J. Bacteriol. **179**: 7369–7378.

Karapinar, M., and Gonul, S.A. 1992. Removal of *Yersinia enterocolitica* from fresh parsley by washing with acetic acid and vinegar. Intl. J. Food Microbiol. **16**: 261–264.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London), **227**: 680–685.

Liao, C.-H. 1989. Analysis of pectate lyase produced by soft rot bacteria associated with spoilage of vegetables. Appl. Environ. Microbiol. **55**: 1677–1683.

Liao, C.-H., and Wells, J.M. 1987. Diversity of pectolytic, fluorescent pseudomonads causing soft rots of fresh vegetables at produce markets. Phytopathology, **77**: 673–677.

Liao, C.-H., Gaffney, T.D., Bradley, S.P., and Wong, L.J.C. 1996. Cloning of a pectate lyase gene from *Xanthomonas campestris* pv. malvacearum and comparison of its sequence relationship with *pel* genes of soft-rot *Erwinia* and *Pseudomonas*. Mol. Plant–Microbe Interact. **9**: 14–21.

Manulis, S., Kobayashi, D.Y., and Keen, N.T. 1988. Molecular cloning and sequencing of a pectate lyase gene from *Yersinia pseudotuberculosis*. J. Bacteriol. **170**: 1825–1830.

Nakajima, H., Inoue, M., Mori, T., Itoh, K.-I., Arakawa, E., and Watanabe, H. 1992. Detection and identification of *Yersinia pseudotuberculosis* and pathogenic *Yersinia enterocolitica* by an improved polymerase chain reaction method. J. Clin. Microbiol. **30**: 2484–2486.

Nguyen-the, C., and Carlin, F. 1994. The microbiology of minimally processed fresh fruits and vegetables. Crit. Rev. Food Sci. Nutr. **34**: 371–401.

Preston, J.F., Rice, D., Ingram, L.O., and Keen, N.T. 1992. Differential depolymerization mechamism of pectate lyase secreted by *Erwinia chrysanthemi* EC16. J. Bacteriol. **174**: 2039–2042.

Robins-Browne, R.M. 1997. *Yersinia enterocolitica. In* Food microbiology: fundamentals and frontiers. *Edited by* M.P. Doyle, L.R. Beuchat, and T.J. Montville. American Society for Microbiology, Washington, D.C. pp. 192–215.

Ruvun, G.B., and Ausubel, F.M. 1981. A general method for site-directed mutagenesis in prokaryotes. Nature (London), **289**: 85–88.

Sambrook, J., Fritsch, E.F., and Maniatis, T.A. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Starr, M.P., Chatterjee, A.K., Starr, P.B., and Buchanan, G.E. 1977. Enzymatic degradation of polygalacturonic acid by *Yersinia* and *Klebsiella* species in relation to clinical laboratory procedures. J. Clin. Microbiol. **6**: 379–386.

Staskawicz, B., Dahlbeck, D., Keen, N., and Napoli, C. 1987. Molecular characterization of cloned avirulence gene from race 0 to race 1 of *Pseudomonas sringae* pv. glycinea. J. Bacteriol. **169**: 5789–5794.

Tacket, C.O., Ballard, J., Harris, N., Allard, J., Nolan, C., Quan, T., and Cohen, M.L. 1985. An outbreak of *Yersinia enterocolitica* infection caused by contaminated tofu (soybean curd). Am. J. Epidemiol. **121**: 705–711.

Von Riesen, V.L. 1975. Polypectate digestion by *Yersinia*. J. Clin. Microbiol. **2**: 552–553.

Witholt, B., Boekhout, M., Brock, M., Kingma, J., Van Heerikhuizen, H., and DeLeij, L. 1976. An efficient and reproducible procedure for the formation of spheroplasts from variously grown *Escherichia coli.* Anal. Biochem. **74**: 160–170.